Final publication is available from Mary Ann Liebert, Inc., publishers https://doi.org/10.1089/thy.2018.0232

Longitudinal characterization of autoantibodies to the thyrotropin receptor (TRAb) during alemtuzumab therapy; evidence that TRAb may precede thyroid dysfunction by many years.

Ilaria Muller[1], Mark Willis[2], Sarah Healy[2], Taha Nasser[2], Samantha Loveless[2], Sara Butterworth[2], Lei Zhang[1], Mohd S Draman[1], Peter N Taylor[1], Neil Robertson[2], Colin M Dayan[1] and Marian E Ludgate[1]

Affiliations

[1] Thyroid Research Group, Division of Infection & Immunity, School of Medicine, Cardiff University,

Address: University Hospital of Wales, Main Building C2 link corridor, Heath Park, Cardiff, CF14 4XN, United Kingdom

[2] Division of Psychological Medicine and Clinical Neurosciences, School of Medicine, Cardiff University

Address: University Hospital of Wales, Main Building C4, Heath Park, Cardiff, CF14 4XN, United Kingdom

Authors' contact information and highest-earned academic degrees

Dr. Ilaria Muller; MD, PhD mulleri4@cardiff.ac.uk +44(0)2920742182

Dr. Mark Willis; MD, MRCP, PhD willismd@cardiff.ac.uk +44(0)2920745403

Dr. Sarah Healy; MD, BSc sarah.healy89@gmail.com +44(0)2920745403

Dr. Taha Nasser; MD smt.nasser@gmail.com +44(0)2920745403

Dr. Samantha Loveless; BSc, PhD lovelesss1@cardiff.ac.uk +44(0)2920745403

Ms. Sara Butterworth; Bsc sara_butterworth@hotmail.co.uk +44(0)2920745403

Dr. Lei Zhang; PhD zhangl14@cardiff.ac.uk +44(0)2920742182

Dr. Mohd Shazli Draman, MD, PhD Dramanyusofms@cardiff.ac.uk +44(0)2920742182

Dr. Peter Nicholas Taylor; MD, MSc TaylorPN@cardiff.ac.uk +44(0)2920742182

Prof. Neil Robertson; MD robertsonnp@cardiff.ac.uk +44(0)2920745403

Prof. Colin Mark Dayan; MD, FRCP, PhD DayanCM@cardiff.ac.uk +44(0)2920742182

Prof. Marian Elizabeth Ludgate; BSc, PhD Ludgate@cardiff.ac.uk +44(0)2920742182

Running title: Longitudinal study of alemtuzumab-related TRAb

Key words: Thyroid Autoimmunity, Graves' Disease, Immune Reconstitution Syndrome, Autoantibodies to the thyrotropin receptor, Alemtuzumab, Thyroid bioassays

ABSTRACT

BACKGROUND

Thyroid autoimmunity, especially Graves' disease or hypothyroidism with positive autoantibodies (TRAb) to the thyrotropin receptor (TSHR), occurs in 30-40% of patients with relapsing multiple sclerosis (MS) following treatment with alemtuzumab (ALTZ). ALTZ therapy therefore provides a unique opportunity to study the evolution of TRAb prior to clinical presentation. TRAb can stimulate (TSAb), block (TBAb) or not affect ("neutral": TNAb) the TSHR function, causing hyperthyroidism, hypothyroidism or euthyroidism, respectively.

METHODS

We conducted a longitudinal retrospective analysis of TRAb bioactivity over a period of 9 years in 45 MS patients receiving ALTZ using available stored serum; 31 developed thyroid dysfunction (TD) and 14 remained euthyroid despite being followed for a minimum of 5 years (NO-TD). The presence of TRAb was evaluated at standardized time points: A) pre-ALTZ, B) latest time available post-ALTZ and before TD onset, C) post-ALTZ during/after TD onset. Serum TRAb were detected by published in-house assays (ihTRAb): flow cytometry (FC) detecting any TSHR-binding TRAb and luciferase bioassays (LB) detecting TSAb/TBAb bioactivity. Purified IgGs were used to verify TSAb/TBAb in selected hypothyroid cases. Standard clinical automated measurements of TRAb (autTRAb), anti-thyroid peroxidase autoantibodies (TPOAb), thyroid stimulating hormone, free-thyroxine and free-triiodothyronine were also collected.

RESULTS

Pre-ALTZ, combined ihTRAb (positive with FC and/or LB), but not autTRAb, were present in 5/16 (31.2%) TD versus 0/14 (0%) NO-TD (p=0.017). Detectable ihTRAb

preceded TD development in 9/28 (32.1%) and by a median of 1.2 years (range 28 days – 7.3 years). Combination testing of ihTRAb and TPOAb at baseline predicted 20% of subsequent cases of hyperthyroidism and 83% of hypothyroidism.

CONCLUSIONS

We present evidence that TRAb measured with custom-made assays can be detected prior to any change in thyroid function in up to a third of cases of ALTZ-related TD. Furthermore, The presence of ihTRAb prior to ALTZ treatment was strongly predictive of subsequent TD. Our findings suggest that a period of affinity maturation of TRAb may precede clinical disease onset in some cases. Combined testing of TPOAb and ihTRAb may increase our ability to predict those who will develop thyroid dysfunction post ALTZ.

INTRODUCTION

Alemtuzumab (ALTZ; Campath-1H) is an anti-CD52 humanized monoclonal which has proven efficacy in relapsing multiple sclerosis (MS) (1). It is administered as a standard treatment regime (two cycles one year apart), with subsequent courses determined by evidence of returning central nervous system inflammatory activity. It causes rapid complement mediated lysis of circulating lymphocytes and profound lymphopenia. Since bone marrow derived lymphoid precursors are unaffected, lymphocyte reconstitution subsequently occurs, which appears to have a beneficial effect. Patterns of lymphocyte re-population vary between patients but circulating B cells return most rapidly and can rise to higher levels than baseline (2), whilst CD4/CD8 T cells numbers recover more slowly, and may never attain pre-treatment levels (3,4). Despite prolonged T cell lymphopenia post-alemtuzumab immune competence is largely preserved and significant infections occur only rarely (5). However 30-48% of patients develop secondary autoimmunity, mainly humoral, 0.5–11 years after treatment (peak incidence 2-3 years) (6-10). The commonest disease (41%) is thyroid autoimmunity (TA) (11), followed by idiopathic thrombocytopenic purpura (1-3%). However, a range of other rare autoimmune disorders including haemolytic anaemia, neutropenia and Goodpasture syndrome have also been reported (1,12-14). The exact pathogenic mechanism for post-ALTZ TA remains unclear, however it is considered to be an "immune reconstitution syndrome", i.e. an autoimmune phenomenon occurring during or after a phase of immune restoration following lymphopenia. This has also been reported in HIV patients following antiretroviral therapy and after bone marrow transplantation (7,8).

Among TA, ALTZ predominantly induces Graves' disease (GD: 63%), followed by hypothyroidism (34%), and rarely transient thyroiditis (11). GD is caused by anti-thyrotropin (TSH) receptor (TSHR) autoantibodies (TRAb) persistently activating the

TSHR (TSHR-stimulating antibodies: TSAb), leading to hyperthyroidism (15). TRAb can also block the TSHR (TSHR-blocking antibodies, TBAb), causing hypothyroidism (16,17), and "neutral" TRAb (which bind the TSHR without affecting thyroid function: TNAb) have been reported in around 12% of subjects with normal thyroid function, 59-84% GD patients (depending on the assay type used), and patients with autoimmune thyroiditis at lower rates (18-20). TNAb seem to bind TSHR but do not activate the cAMP signaling cascade, which is the principal pathway leading to thyroid hormones synthesis; however they may be able to trigger alternative and multiple signaling cascades having complex downstream effects, including oxidative stress (20).

In spontaneous TA, TBAb account for a minority of cases of hypothyroidism (around 9-10%) (16,21), the remainder being due to lymphocyte-mediated damaging of the thyroid, as in classical Hashimoto's thyroiditis (22). Autoantibodies to thyroid peroxidase (TPOAb) are the hallmark of such autoimmune thyroiditis, however they are very often positive in GD also, indicating that in TA the self-tolerance breakdown involves multiple thyroid antigens (23). Surprisingly, TRAb are positive in 50.0%-76.7% of patients with post-ALTZ hypothyroidism (9,10), with TBAb representing a common mechanism of post-ALTZ hypothyroidism in a recent analysis (10).

TPOAb are very common in the general population (up to 20%) (24,25), and have been identified as a predictive marker of TD subsequent to ALTZ (9). In particular, 69% of MS subjects TPOAb positive before ALTZ developed subsequent TD, compared to 31% of TPOAb negative subjects. However, 85% patients who later developed TD were TPOAb negative at baseline, indicating that TPOAb status alone has limited value in risk stratification in the majority of patients (9).

The longitudinal study of ALTZ-treated patients provides a rare opportunity to study TRAb prevalence and biological function prior to disease "triggering" in patients

who develop GD. The automated TRAb assays (autTRAb) used in clinical diagnostics are unable to distinguish TSAb/TBAb (26); as a result several groups including ours have developed in-house bioassays able to detect TSAb (27,28) and TBAb (21,29), as well as TNAb (19). We postulated that TRAb, in particular TNAb, pre-existing before ALTZ may be the precursors of the TSAb and TBAb that subsequently develop by somatic hypermutation and affinity maturation in B cells (30,31). Detection of low titre or low affinity TSAb/TBAb or the presence of TNAb prior to ALTZ therapy, in combination with TPOAb testing, may also increase our ability to predict thyroid dysfunction after ALTZ.

In addition we used the in-house TRAb bioassays (ihTRAb) to analyze TRAb bioactivity arising after ALTZ therapy, which has so far only been described in spontaneous TA (21,28,32). In a recent UK study conducted in collaboration between Cambridge and Cardiff we have introduced TSAb/TBAb analysis in post-ALTZ TA, however this was limited to only a few patients affected with hypothyroidism or "fluctuating" GD, defined as multiple alternate phases of hyperthyroidism and hypothyroidism (10). In the present study we extended this analysis to all available cases, including a third different in-house assay to detect TSHR-binding TRAb independently from their bioactivity (19).

MATERIALS AND METHODS

Patients and sera

Blood samples from Welsh MS patients consenting to research have been consecutively collected for research purposes from 2006 (REC# 05/WSE03/111), and stored within the Welsh Neuroscience Research Tissue Bank (WNRTB: Cardiff, UK, REC# 14/WA/0073). Blood samples were processed within 3 hours of collection

following a standardized protocol including spin at 4500 rpm for 10 minutes at +4°C. Serum and plasma were subsequently aliquoted and stored at -80°C.

Sera of 45 patients affected with relapsing MS treated with ALTZ with longitudinal samples between August 2006 to October 2015 were identified including samples from 31 consecutive subjects with post-ALTZ thyroid dysfunction (TD). Samples from 14 patients who had not developed TD (NO-TD) were also selected based on the availability of serum before ALTZ, and clinical follow-up of ≥5 years, in order to exclude cases of late TD onset (11). Sera from pre-specified time-points were requested for TD and NO-TD groups (Figure 1): A) first available pre-ALTZ time; B) the latest time available post-ALTZ and before the TD onset; C) post-ALTZ at the TD onset, or alternatively the earliest subsequent time available (TD only).

All patients were treated with ALTZ at the University Hospital of Wales (UHW) in Cardiff, UK, and followed up both at UHW and local Welsh hospitals. ALTZ was administered intravenously 5 consecutive days for the first cycle, with the majority of subjects receiving a second cycle (3 consecutive days) 12 months later; in some patients further doses were given at least one year apart, depending on clinical and radiological outcomes. The date of the first ALTZ administration within our patient cohort ranged from April 2002 to November 2012; the initiation dose was 24-30 mg/day prior to 2006, then reduced to 12 mg/day. Since blood collection for research purposes commenced only in 2006, this explains why time-point A is missing in several patients.

Information about patients' age, TSH, free-thyroxine (FT4), free-triiodothyronine (FT3), TPOAb, TRAb determined by automated assays, thyroid treatment, and number of ALTZ treatments were collected, when available. Demographic information and detailed longitudinal clinical information was available for all patients, with last update in February 2018.

Luciferase bioassays (TSAb/TBAb)

In-house luciferase bioassays (LB) to detect TSAb and TBAb were performed using a Chinese Hamster Ovary (CHO) cell line stably transfected with the human TSHR and a cAMP responsive luciferase reporter (pA3Luc), as previously described (Lulu*) (27,29). Briefly, cells were seeded at $2x10^4$ cells/well in 96-well plates in Ham's F12 containing 10% fetal calf serum, and switched to Ham's F12 containing 10% charcoal stripped calf serum the day before the assay. In the assays cells were incubated for 4 hours at 37°C in 5% CO2 in air with whole human serum (1:10 dilution) in serum-free medium (SFM: Ham's F-12 supplemented with 2.5% sodium bicarbonate) for the TSAb assay, and SFM containing 1 mU/ml bovine TSH (bTSH; Sigma-Aldrich Company Ltd., Poole, UK) in the TBAb assay. Cells were also incubated with SFM alone as negative control, and 5 mU/ml bTSH and 0.2 ng/µl M22 human monoclonal Ab to TSHR (RSR, Cardiff, UK) as positive controls. Cells were finally lysed, and the luciferase activity measured using commercially available kits (Promega, Madison, USA) and a luminometer machine (Glomax®-Multi Detection System, Promega).

Randomly selected sera from 9 euthyroid participants from the Controlled Antenatal Thyroid Screening II (CATS II) study (33,34) were used as euthyroid pool in both TSAb/TBAb assays; they were all adult women (mean age ± standard deviation = 40.8 ± 5.3 years) with normal thyroid function and negative for TPOAb.

In the TSAb assay, CHO cells transfected with pA3Luc only (Zulu) were used in parallel to Lulu*. The considered positivity cut-off was a stimulation index (SI) >1.5 calculated with the following formula:

$$SI = \frac{\text{light patient sample Lulu* / light patient sample Zulu}}{\text{light euthyroid pool Lulu* / light euthyroid pool Zulu}}$$

The TBAb assay positivity cut-off was an inhibition index (InI) >20% as

previously determined (formula A) (29) using Lulu* cultured with 1 mU/ml bTSH:

$$InI = 100 \times \frac{(1 - \text{light patient sample})}{\text{light euthyroid pool}}$$

In order to exclude interference of high serum TSH levels with our in-house TSAb/TBAb serum assay, especially among hypothyroid patients, experiments were repeated using IgG (amount equivalent to 1:10 serum dilution) in place of serum; if results were discordant we counted those using IgGs. IgGs were purified from selected serum samples with the Melon Gel IgG Purification Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Briefly, serum samples were diluted 1:10 and the diluted serum was added to a spin column containing the Melon Gel resin. After 30 minutes incubation, the purified IgGs were collected in the flow through by centrifugation of the spin column, and the IgG concentration measured by ultraviolet optical absorption at 280 nm with a NanoDrop™ Lite spectrophotometer (Thermo Scientific). All IgG purified samples were promptly used for downstream analysis, or aliquoted and stored at -20 °C.

Flow Cytometry (TSHR-binding TRAb)

In order to reduce the high non-specific background staining due to human antibodies recognizing and/or cross-binding to surface CHO proteins, a serum pre-adsorption step using Zulu cells was performed as previously described (35).

Flow cytometry (FC) detection of TSHR-binding TRAb (FC-TRAb) in pre-adsorbed sera was then performed using CHO cells expressing the glycosylphosphatidylinositol (GPI)-anchored TSHR extracellular domain (CHO-TSHR), as previously described (19). As minor protocol modifications, 1:100 goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (Life Technologies) and 1:1000 LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Invitrogen) were used as second conjugated-antibody and viability dye, respectively (35). Zulu cells were used as CHO control cell

line not expressing TSHR. The fluorescence of 10,000 cells/tube was assayed by BD FACSCanto II flow cytofluorometer, FACSDiva Software (BD Biosciences, San Jose, USA); no FITC (TRAb) and Apc-Cy7 (LIVE/DEAD®) channels compensation was needed (500-520 nm and 633-750 nm excitation-emission peaks wavelengths respectively)(35).

Flow Cytometric data were analyzed using FlowJo 8.8.6 Software (TreeStar Inc., Ashland, USA), and damaged or dead cells (Apc-Cy7 positive) gated and excluded from analysis (35). The geometric mean FITC fluorescence intensity values of CHO-TSHR and Zulu cells were compared for all sera and the Kolmogorov–Smirnov univariate two-sample test was used to obtain the greatest difference between the two histograms, quoted as D value (D) (36). Cut-off values were defined based on the mean D +2 SD of individual pre-adsorbed sera from 9 healthy women from the CATS II study (33,34) used as controls; all values higher than this were considered positive (FC-TRAb+) (35).

Automated Laboratory Measurements

Automated TRAb (autTRAb) were measured with the Brahms Diagnostika Lumitest TRAK assay (Germany; Reference Ranges IU/L = Negative <1, Borderline 1–1.5, Positive >1.5) until January 2014, then using the Roche Cobas® e411 assay (Switzerland; Reference Ranges IU/L = Negative <0.9, Borderline 0.9–1.6, Positive >1.6). According to Thermoscientific, human TSH does not interfere with TRAb measurement in the Lumitest TRAK assay, up to TSH values of at least 500mU/L. UHW Biochemistry Department also run specific cross-reactivity tests using patient serum with a TSH concentration of 179 mU/L, confirming no interference with neither Brahms nor Roche TRAb assays.

TPOAb, TSH, FT4 and FT3 analyses were performed using an ADVIA Centaur automated immunoassay analyser (Bayer plc, UK) until 31/05/2010, followed by

Chemiluminescent Microparticle Immunoassay methods by the ARCHITECT® System (ABBOTT Laboratories, USA) until the end of the observation period. Supplemental Table 1 summarizes the changes of reference ranges occurred during this time period.

Definitions of Thyroid Function

All 45 patients included in the study were euthyroid when receiving the first ALTZ treatment, and had no clinical history of thyroid disease. The time of TD onset was defined as the first alteration of the thyroid function defined as persistent (i.e. detectable in consecutive blood tests at least 3 months apart) and/or significant (i.e. requiring immediate thyroid treatment). Hyperthyroidism was defined as low TSH with or without raised FT4/FT3 levels; hypothyroidism was defined as raised TSH with or without low FT4/FT3 levels.

Thyroid diagnosis was defined as:

I) GD: TRAb+ hyperthyroidism

II) Fluctuating GD: TRAb+ cases with multiple alternate phases of hyperthyroidism and hypothyroidism, not explained by overtreatment or poor treatment compliance

III) TRAb+ hypothyroidism

IV) Chronic autoimmune thyroiditis (37): persistent hypothyroidism (≥ 6 months) with positive TPOAb and negative TRAb

V) Subacute thyroiditis: transient hyperthyroidism, hypothyroidism or both with TD lasting in total <6 months, TRAb negative, with or without TPOAb

VI) TPOAb-/TRAb- hypothyroidism: persistent hypothyroidism (≥ 6 months) with negative TPOAb and TRAb

Statistical Analysis

According to the TRAb prevalence in the general population of 12% (19), our *a priori* power calculation indicated 12 versus 12 subjects required to provide 80% power to

detect a 5-fold TRAb prevalence (60%) in patients that will later develop ALTZ-induced thyroid dysfunction, with a 0.05 significance level (two-tailed).

Presence of TRAb at different time-points was compared between TD and NO-TD groups using the Fisher Exact Text, considering $p<0.05$ as significance level. As explorative analysis, positivity of TPOAb and TRAb measured with automated assays was also considered.

Fisher exact test and t-test were used also to compare the characteristics of TD and NO-TD groups, considering $p<0.05$ as significance level.

RESULTS

Patients

The date of first ALTZ treatment ranged from 2002 to 2012 (median 2008), and the mean ± SD follow-up was 9.0 ± 2.5 years post-ALTZ (range: 4.3 – 14.0 years). The TD group comprised patients showing post-ALTZ hyperthyroidism (n=19) or hypothyroidism (n=12) as first clinical manifestation (TD onset).

Table 1 summarizes the characteristics of TD and NO-TD groups; no significant differences were detected between the different groups. Before TD onset (time-points A and B) all patients were euthyroid and free of persistent thyroid function abnormalities. Note that at time-point C (TD group) many patients who developed thyroid dysfunction were already on thyroid medication: a detailed description of their treatments and outcomes has been reported elsewhere (10).

Combined in-house TRAb (ihTRAb) results at all time-points

We compared the overall results obtained with the three different ihTRAb assays (FC-TRAb, LB-TSAb, LB-TBAb) at all time-points in TD and NO-TD groups. Due to the retrospective nature of this study, sera from some time-points were unavailable for the TD

group (Table 1). As shown in Figure 2, at time-point A (before ALTZ) 5/16 (31.2%) TD patients were found to be ihTRAb positive (ihTRAb+), compared with 0/14 (0%) NO-TD patients (p=0.017). Following ALTZ, 6/25 (24.0%) TD patients were ihTRAb+ at time-point B (before TD onset); as expected, at time-point C (during or after TD onset) ihTRAb+ cases markedly increased to 18/29 (62.1%). This prevalence is likely to be underestimated, considering the late average collection time of time-point C compared with disease onset (Table 1). Among NO-TD patients, 4/14 (28.6%) were ihTRAb+ at time-point B. When splitting the overall ihTRAb+ results according to the TD subtype at onset (hyperthyroidism or hypothyroidism), time-point A ihTRAb were predominantly positive in those who subsequently developed hypothyroidism (4/6: 66.7%) rather than hyperthyroidism (1/10: 10%), p=0.036. It is worth noting that two initially hypothyroid ihTRAb+ patients subsequently showed a fluctuating thyroid function and were classified as fluctuating GD.

Time-point A: predictors of post-ALTZ TD

To validate TRAb as an independent predictor of ALTZ-induced TD, we compared ihTRAb results with autTRAb and TPOAb data at time-point A (Table 2). Pre-ALTZ ihTRAb and TPOAb had a very similar predictive value for future TD development. When ihTRAb and TPOAb testing were combined together, 7/16 (43.8%) TD patients were positive, versus 0/14 of NO-TD group (p=0.007); in particular 83.3% hypothyroid and 20% hyperthyroid cases were predicted, versus 50% and 20% respectively when considering TPOAb alone (Table 2, last two columns). Detailed TRAb and/or TPOAb predictive values, sensitivity and specificity have been reported in supplemental Table 2.

Considering this from a different perspective, TD developed in 7/7 (100%) baseline ihTRAb and/or TPOAb positive patients, versus 9/23 (39.1%) baseline ihTRAb and/or TPOAb negative patients (p=0.007).

AutTRAb were positive before ALTZ in only 1 patient of the TD hyperthyroid subgroup (14.3%) and none in the hypothyroid group, suggesting that autTRAb do not appear to be a useful predictive marker of subsequent TD development.

In depth analysis of ihTRAb+ cases

Table 3 reports in more detail the ihTRAb+ cases only, describing the different ihTRAb subtypes in comparison with autTRAb, TPOAb, and TSH results, when available. Here the hyperthyroid group was further subdivided into classic hyperthyroid GD and fluctuating GD. At time-point A ihTRAb+ cases as expected were predominantly TNAb (3/5: 60%), defined as FC-TRAb+ but both LB-TSAb/TBAb negative (Table 3).

At time-point B, ihTRAb+ cases were represented by a similar proportion of TNAb, TSAb and TBAb (Table 3). In combination, TNAb or TSAb/TBAb preceded TD onset in 9 cases (32.1%, considering a total of 28 TD patients with time-point A and/or B available) with an interval before TD onset of a median of 1.2 years (range 28 days – 7.3 years).

At time-point C (Table 3), as expected all ihTRAb+ hyperthyroid GD and fluctuating GD patients were also autTRAb+, confirming a GD diagnosis. Among ihTRAb+ hyperthyroid and fluctuating GD cases, FC-TRAb was the most sensitive assay with 13/14 (92.9%) positive, versus 9/14 (64.3%) of TSAb. TBAb were positive in 3/10 (30%) purely hyperthyroid ihTRAb+ GD patients. As expected fluctuating GD cases had a documented TSAb/TBAb coexistence in 2/4 (50%) cases (IDs 35, 42); the other two cases were positive for TSAb only (IDs 1, 7).

Among the whole hypothyroid group (n=10), 4 (40%) were ihTRAb+ at time-point C, in particular 2/4 (50%) FC-TRAb+ and 2/4 (50%) both FC-TRAb+ and TBAb+; autTRAb results were concordant (Table 3). Surprisingly, both ihTRAb+ hypothyroid patients at time-point A (IDs 15, 37) resulted ihTRAb negative at time-point C.

Final thyroid diagnosis

TPOAb titres measured anytime post-ALTZ were available in 17/19 (89.5%) of hyperthyroid group, and were positive in 15/17 (88.2%) cases; in fact two TPOAb negative GD patients at time-point C (Table 3) later became TPOAb positive, for example ID 27. Anytime post-ALTZ, TPOAb were positive in 11/12 (91.7%) of hypothyroid patients. None of the 14 NO-TD patients developed post-ALTZ TPOAb.

According to their clinical course, TD patients were classified as pure hyperthyroid GD (n=17), fluctuating GD (n=4), and hypothyroid patients (n=10). AutTRAb were positive in 17/17 (100%) GD and 4/4 (100%) fluctuating GD. Taking our ihTRAb results into account to better define the final thyroid diagnosis among the 31 TD patients according to the criteria given in the methods identified 17 (54.8%) GD, 4 (12.9%) fluctuating GD (2 started with hypothyroidism, 2 with hyperthyroidism), 4 (12.9%) TRAb+ hypothyroidism, 3 (9.7%) chronic autoimmune thyroiditis, 2 (6.5%) TPOAb+ subacute thyroiditis, 1 (3.2%) TPOAb-/TRAb- hypothyroidism.

DISCUSSION

We have described for the first time the biological function of TRAb in a longitudinal cohort of patients developing ALTZ-induced thyroid dysfunction (TD) using three different in-house TRAb assays (ihTRAb). Importantly, as a result of a structured monitoring and sampling process for patients with MS in south Wales and suitable for ALTZ treatment, serum was available before the onset of TD enabling us to describe how

and when TRAb become positive in patients with ALTZ-induced TD. This setting is unique, as serum is not generally available before disease onset in sporadic GD. Interestingly, serum ihTRAb, but not TRAb detected with standard automated assays (autTRAb), were detected before ALTZ in one third of patients who later developed TD, and in none of those who remained free of TD (NO-TD) over a minimum follow-up period of 5 years. The appearance of ihTRAb was detected a mean of 1.2 years (range 28 days – 7.3 years) prior to the development of thyroid dysfunction. We believe this is the first report of the detection of TRAb prior to the onset of ALTZ-induced TD. Similar findings have been previously described for spontaneous TD in a retrospective study showing progressively increasing TRAb positivity, as well as TPOAb and anti-thyroglobulin antibodies, in patients who will later develop GD. In particular TRAb positivity increased from 2% at 7 years before diagnosis to 55% at diagnosis, with intermediate percentages of 7% and 20% at -5 and -2 years, respectively (38).

Furthermore, in our study for the first time we provided details about TRAb biological function over time. We have previously reported the presence neutral TRAb (TNAb), detected using flow cytometry, in healthy euthyroid subjects, but without any follow-up clinical data to indicate whether they later did develop TD (19). Information similar to our data in ALTZ-induced disease are difficult to collect in the setting of spontaneous autoimmune TD, requiring very large and long-term cohort studies. The fact that the rates of TD post ALTZ are much higher than generally seen in MS, suggests that the two settings are not necessarily comparable, however the principle that autoimmunity to the TSHR may precede TD by many months or years applies to both ALTZ-induced (this study) and spontaneous forms as reported by others (38). Note that the wide range of pre-TD intervals (28 days – 7.3 years) is partly a consequence of the retrospective nature of this study, not providing systematic and identical time-points for all patients. Future

prospective studies are needed to precisely define how long ihTRAb may precede the onset of TD in some cases.

In cases of TRAb positivity pre-dating TD, we hypothesize that TSHR-reactive B cell clones may undergo progressive antigen-driven affinity maturation by somatic hypermutation within germinal centres, and finally generate high affinity stimulating (TSAb) or blocking (TBAb) TRAb. The phenomenon of multiple different pathogenic TRAb arising from single B cell clones by somatic hypermutation has already been described in mouse models of GD (30,31). In this context, our finding that ihTRAb more commonly preceded hypo- than hyperthyroidism is interesting, but may reflect that once a stimulatory TSAb-secreting clone develops, TD follows rapidly whereas it may take longer for TBAb to achieve clinically relevant inhibition of thyroid function such that TSH levels rise. Our observations are in accordance with previous evidence that TSAb are potent at low concentrations, therefore inducing hyperthyroidism rapidly after their appearance (23), while TBAb levels needed to trigger hypothyroidism are usually much higher than TSAb levels inducing hyperthyroidism (26). Further prospective studies with large numbers of subjects should clarify this. It also has to be mentioned that we did not sub-classify TD patients into subclinical and overt disease since the vast majority of patients diagnosed with subclinical disease went on to develop overt thyroid dysfunction, or were treated immediately after diagnosis, preventing the possible evolution to overt disease.

Although it is understandable that TNAb can exist without altering thyroid function, it is less clear how this is possible with TSAb and TBAb. Possible explanations for TSAb/TBAb positive cases in euthyroid patients are: i) they are low-affinity, therefore not able to exert a significant function on TSHR activity with clinical consequence; ii) the *in vitro* assays in some cases do not reflect the different and more complex human thyroid

environment, providing slightly different results from *in vivo*. For example luciferase bioassays use bovine and not human TSH, CHO cells instead of human thyrocytes, and only the cAMP pathway is investigated. The same observations about TNAb or low-affinity TSAb/TBAb apply to 28.6% euthyroid NO-TD patients developing post-ALTZ TRAb; in the future they might remain positive with no long-term clinical consequences, or might develop late onset TD.

TD post ALTZ is often delayed by several years and the ability to reliably predict those at risk would allow targeted monitoring and possibly early intervention or prevention. TPOAb are already known to identify subjects at risk, with 69% of individuals TPOAb+ at baseline developing TD. However, TPOAb testing only detects around 15% of all future cases of TD post-ALTZ (9). Our data suggests that custom-made TRAb testing in combination with TPOAb testing at baseline might increase this to predicting around 20% of hyperthyroid cases and 80% of hypothyroid cases. Interestingly, in 2 hypothyroid patients pre-ALTZ ihTRAb positivity was no longer detectable at the time of disease onset, suggesting that TRAb titres fluctuate over time and are not always detectable. Furthermore, in these cases they also might have become spontaneously negative, and destructive thyroiditis might represent the sole mechanism of hypothyroidism.

The analysis of ihTRAb proved less valuable for predicting the disease course after the onset of TD than expected. For example, not all subjects who developed hypothyroidism or GD with fluctuating course had detectable TBAb. Several explanations are possible: i) non-optimal timing of time-point C, often several months after the disease onset and the commencing of anti-thyroid treatments, usually associated with TRAb titres decrease and negativization; ii) TSAb/TBAb levels might fluctuate over time, not being always positive at the same time; iii) TBAb might interact with the TSHR with a lower

affinity compared with TSAb, and therefore might be masked by TSAb coexistence in our biological assays. Similarly, TBAb false positive cases have been described due to the concomitant presence of TSAb; if TSAb act as weak agonists, they interfere with the bTSH in the TBAb assay resulting in a signal reduction. In general, TSAb/TBAb coexistence can be challenging to demonstrate due to their mutual interference, depending on relative concentrations, affinities and potencies, varying over time. Sometimes serum serial dilutions are needed to properly distinguish between the two TRAb populations (26).

However our findings show that around 40% of hypothyroidism post ALTZ is TBAb mediated, as suggested in previous studies (9,10); this is nonetheless substantially higher than reports in spontaneous disease (around 10%) (16,21). By contrast, 91.7% (11/12) of hypothyroid subjects were TPOAb positive, consistent with TBAb negative hypothyroidism post-ALTZ still being autoimmune in the majority of cases, but perhaps cell-mediated. However, it was notable that autTRAb were detectable in many subjects who developed hypothyroidism or a switching course as well as all those with hyperthyroidism. Currently, autTRAb measurement is recommended only in patients developing hyperthyroidism; if our observations are confirmed in larger prospective studies, autTRAb testing should probably be extended to all cases of post-ALTZ TD, including hypothyroidism, since they appear to predict a more complex clinical course (i.e. possibility of thyroid function switching) requiring close observation.

The strength of our study is the long follow-up to define outcome ($\geq$ 5 years where no TD is reported) and the wide range of thyroid autoantibody assays used. However, ALTZ has only recently been licensed for use in relapsing/remitting MS (since 2014) and hence ALTZ-induced TD is currently not very common, especially cases with the long follow-up required to define outcome. As a result, our cohort is relatively small (n=45) and this is

a limitation. Furthermore, due to the retrospective nature of the study, serum was not available at all time-points in the whole cohort, and in particular, samples at the time of TD onset were not always available. However we believe our finding that TRAb can precede disease onset and are associated with subsequent TD is robust as our numbers were consistent with our *a priori* power calculations.

In conclusion we have observed that TRAb can precede TD by many years and, if present before ALTZ, can increase the risk of subsequent development of TD. Future prospective studies are needed to determine the exact value of baseline and follow-up TRAb testing in subjects treated with ALTZ and the most valuable assay to use. Such studies, as well as large cohort studies in spontaneous thyroid autoimmunity may also be used to investigate and define the process of affinity maturation in TRAb further. Now that ALTZ is licensed for the treatment of relapsing/remitting MS in more than 60 countries, the available case load for prospective studies is likely to substantially increase and make at least the studies in ALTZ induced disease feasible.

ACKNOWLEDGMENTS

This study has been supported by the Society for Endocrinology (SfE) Early Career Grant to Dr. Ilaria Muller.

The authors are also grateful to the Welsh Neuroscience Research Tissue Bank (WNRTB: Cardiff, UK) for providing the human sera used in the present study, and to the patients providing their consent for research purposes.

DISCLOSURE STATEMENT

Dr. Muller reports grants from the Society for Endocrinology (SfE) during the conduct of the study. No other competing financial interests exist.

CORRESPONDING AUTHOR

Dr. Ilaria Muller, MD, PhD

Thyroid Research Group, Division of Infection & Immunity, School of Medicine, Cardiff University

Main building Room 256 C2 Link Corridor, University Hospital of Wales, Heath Park, CF14 4XN, Cardiff, United Kingdom (UK)

Phone: +44(0)2920742182, +44(0)2920745409

Fax: +44 (0)2920744671

Email address: mulleri4@cardiff.ac.uk

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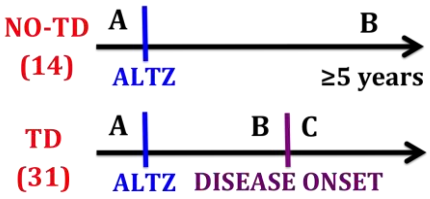
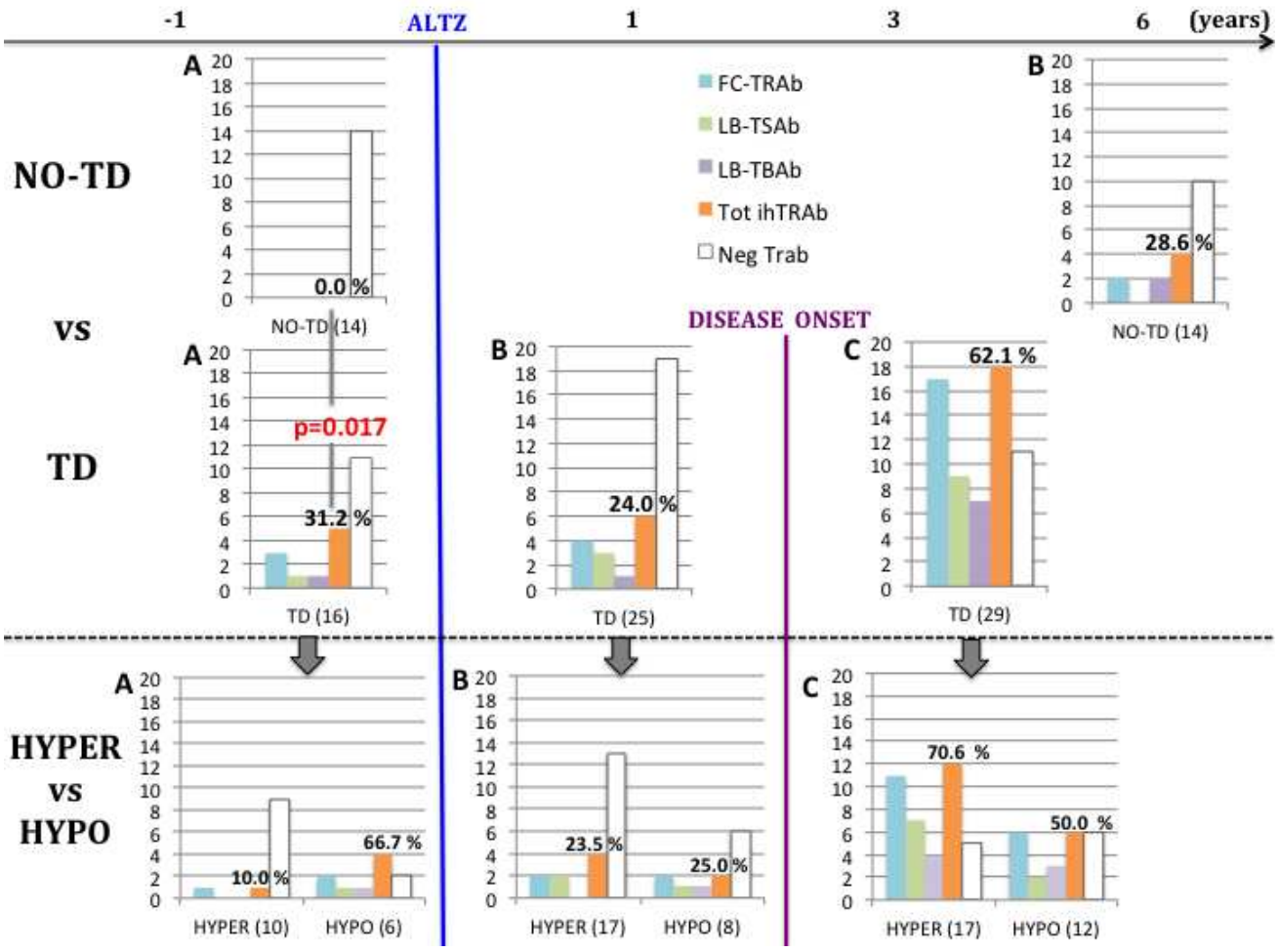
FIGURES

Figure 1: Serum time-points



ALTZ: first alemtuzumab treatment. TD: patients developing thyroid dysfunction. NO-TD: patients not developing thyroid dysfunction.
A: first available time before ALTZ (both TD and NO-TD). B: post ALTZ, first available time before the onset of thyroid dysfunction (TD) or the latest available time post-ALTZ (NO-TD). C: post ALTZ during the onset of thyroid function abnormalities, or in alternative the earliest available time after it (TD only).

Figure 2: ihTRAb positive results at all time-points



Cross-sectional results of all available sera at pre-specified time-points (see Figure 1) analysed with in-house assays to detect autoantibodies to the thyrotropin receptor (ihTRAb), obtained in patients developing thyroid dysfunction (TD) and patients not developing any thyroid dysfunction (NO-TD). Below the dashed line TD patients were further sub-grouped into hyperthyroidism (HYPER) or hypothyroidism (HYPO) as first clinical manifestation. Numbers in brackets indicate the total number of available sera for each time-point and patient subgroup.

A: time-point A = before the first treatment with alemtuzumab (ALTZ). B: time-point B = latest available time post-ALTZ and before TD onset, when applicable. C: time-point C (TD only) = post ALTZ during the onset of thyroid function abnormalities, or in alternative the earliest available time after it.

FC-TRAb (azure) = TRAb detected by flow cytometry. LB-TSAb (green) = Stimulating TRAb detected by luciferase bioassays. LB-TBAb (purple) = Blocking TRAb detected by luciferase bioassays. Tot ihTRAb (orange) = positive FC-TRAb and/or LB-TSAb and/or LB-TBAb; percentages refer to this column. Neg TRAb (white) = ihTRAb negative results with all the three FC, LB-TSAb and LB-TBAb techniques.

TABLES

Table 1: Patients' characteristics

		TD			NO-TD (n=14)
		1st manifestation: Hyperthyroidism (n=19)	1st manifestation: Hypothyroidism (n=12)	Overall (n=31)	
Female: n (%)		15 (78.9%)	8 (66.7%)	23 (74.2%)	10 (71.4%)
Age (years) at 1st ALTZ: mean (SD)		32.0 (7.2)	37.0 (10.6)	33.8 (8.9)	35.0 (9.5)
Tot n ALTZ treatments received: mean (SD)		1.8 (0.7) [1]	2.2 (1.0) [1]	2.0 (0.8)[1]	2.8 (0.8) [2]
years from 1st ALTZ to TD onset: mean (SD), median (range)		3.1 (2.2) 2.0 (1.0-8.7)	3.2 (2.1) 3.0 (0.8-7.3)	3.0 (1.9) 2.7 (0.8-8.7)	NA
Time-point A[3]: median (range)	days before 1st ALTZ	171.5 (26-700)	141 (0-418)	162.0 (0-700)	121.5 (0-375)
Time-point B[4]: median (range)	years after 1st ALTZ	1.4 (0.3-7.8)	2.5 (0.6-6.6)	1.63 (0.3-7.8)	6.2 (5.1-8.1)
	days before TD onset	192.0 (56-680)	179 (28-583)	192.0 (28-680)	NA
Time-point C[5]: median (range)	years after 1st ALTZ	3.1 (1.3-8.8)	3.4 (0.8-9.0)	3.1 (0.8-9.0)	NA
	days after TD onset	89.0 (0-794)	82.5 (0-829)	89.0 (0-829)	NA

ALTZ = alemtuzumab treatment. n = number. NA = Not Applicable. TD = thyroid dysfunction (abnormal thyroid hormones). TD onset = time of the first TD defined as persistent (i.e. detectable in consecutive blood tests at least 3 months apart) and/or significant (i.e. requiring to immediate start a thyroid treatment).

Fisher exact test (gender distribution) and t-test (other variables) excluded significant differences between the groups, when comparable (p= ns).

[1]= until TD onset

[2]= until the end of observational period (time-point B)

[3]= Time-point A serum was available in 10 hyperthyroid, 6 hypothyroid (16 overall TD) and 14 NO-TD patients.

[4]= Time-point B serum was available in 17 hyperthyroid, 8 hypothyroid (25 overall TD) and 14 NO-TD patients.

[5]= Time-point C serum was available in 17 hyperthyroid, 12 hypothyroid (29 overall TD) patients.

Final publication is available from Mary Ann Liebert, Inc., publishers https://doi.org/10.1089/thy.2018.0232

Table 2: Time-point A: Predictive value of baseline TRAb versus TPOAb

	No of Patients	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPOAb	TPOAb and/or TRAb
HYPERTHYROID	1						
	1				NA		
	2				NA		
	6						
	Tot: 10	**1/10** (10%)	**0/10** (0%)	**0/10** (0%)	**1/7** (14.3%)	**2/10** (20%)	**2/10** (20%)
HYPOTHYROID	1				NA		
	1						
	1*				NA		
	1*						
	1						
	1						
	Tot: 6	**2/6** (33.3%)	**1/6** (16.7%)	**1/6** (16.7%)	**0/4** (0%)	**3/6** (50%)	**5/6** (83.3%)
NO-TD	4				NA		
	10						
	Tot: 14	**0/14** (0%)	**0/14** (0%)	**0/14** (0%)	**0/10** (0%)	**0/14** (0%)	**0/14** (0%)

Cross-sectional results of all available sera at time-point A (before alemtuzumab treatment; see Figure 1) for autoantibodies to the thyrotropin receptor (TRAb) and autoantibodies to thyroid peroxidase (TPOAb), obtained in patients developing subsequent hyperthyroidism or hypothyroidism as first clinical manifestation, and patients not developing any thyroid dysfunction (NO-TD) following alemtuzumab treatment.

White cells = negative TRAb/TPOAb results. Colored squares represent positive results: azure = TRAb detected by flow cytometry (FC-TRAb); green = stimulating TRAb detected by luciferase bioassays (LB-TSAb); purple = blocking TRAb detected by luciferase bioassays (LB-TBAb); grey = TRAb detected by automated systems (Aut-TRAb); yellow = TPOAb (automated assay); red = TRAb (any test) and/or TPOAb. NA = Not Available.

* Fluctuating Graves' disease (GD) presenting hypothyroidism as first clinical manifestation.

Table 3: TRAb, TPOAb and TSH status in patients with positive in-house TRAb assays (ihTRAb+)

Hyperthyroid patients (GD)

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time							Time-point C: Post thyroid dysfunction						
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
8	1.05	0.33[2]	0.99	-8.58	2.2	80[A]	159	1.34	0.45[2]	0.98	-12.98	1.9	225[A]	238	< 0.01	0.30[2]	1.38	8.56	>40	66[B]
4	1.86	0.15[1]	1.12	-7.95	NA	<2[B]	105	0.27	0.24[3]	1.54	-4.72	<1	58[A]	35	< 0.01	0.57[1]	4.24	3.52	>40	<2[B]
5	0.96	0.28[2]	0.92	-28.85	<1	<2[A]	418	1.01	0.49[2]	1.12	-8.40	<1	NA	0	< 0.01	0.44[2]	0.76	21.03	12.0	403[B]
2	0.4	0.06[1]	1.34	-22.75	NA	<2[B]	387	^	NA	1.00	-11.74	0.3	NA	166	#	0.49[1]	0.92	16.26	38.0	352[B]
6	1.02	0.18[2]	0.87	-0.76	<1	18[A]	302	1.27	0.13[2]	0.75	4.71	0.6	<2[B]	22	< 0.01	0.75[2]	2.78	0.19	17.6	14[B]
27	2.37	0.21[3]	1.10	4.57	0.3	<2[B]	NA	NA	NA	NA	NA	NA	NA	182	3.87	0.31[3]	1.12	26.84	2.4	9[B]
40	NA	NA	NA	NA	NA	NA	334	0.86	0.28[5]	1.65	-13.88	1.7	13[A]	37	< 0.01	0.53[5]	3.96	-21.73	33.6	2[B]
36	NA	NA	NA	NA	NA	NA	680	1.45	0.06[5]	1.46	-6.20	0.3	56[A]	0	< 0.01	0.65[5]	3.04	-22.92	>40	22[B]
38	NA	NA	NA	NA	NA	NA	142	4.10	0.11[4]	0.78	-48.12	0.5	36[A]	0	< 0.01	0.34[4]	1.32	-77.57	6.9	124[A]
41	NA	NA	NA	NA	NA	NA	294	1.00	0.02[4]	1.15	-43.97	<1	5[B]	177	< 0.01	0.87[4]	2.97	48.78	>40	728[A]

ID 2: missing TSH values for both time-points B (^) and C (#), so the closest TSH results are provided:
^ previous TSH= 0.36 mU/L (233 days before); next TSH <0.02 mU/L (202 days after, during a transient subclinical hyperthyroidism phase lasted <3 months).
# previous TSH <0.02 mU/L (166 days before, same day of thyroid dysfunction onset); next TSH= 17.81 mU/L (55 days after, during carbimazole treatment).

Fluctuating GD

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time							Time-point C: Post thyroid dysfunction						
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
1	1.40	0.16[1]	0.6	32.58	<1	26[A]	118	0.53	0.18[2]	0.95	-20.00	<1	31[A]	829	1.76	0.38[1]	2.46	-16.31	>40	491[A]
35	2.14	0.23[4]	0.88	8.98	<1	3[B]	583	□	0.23[4]	1.19	-13.41	0.6	NA	0	159.68	0.76[4]	3.34	38.02	>40	442[B]
7	0.89	0.05[2]	1.21	-5.97	<1	44[A]	441	0.34	0.09[2]	1.02	4.57	NA	<2[B]	196	69.25	0.68[2]	3.46	11.93	>40	82[B]
42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	197	52.15	0.78[4]	2.16	40.90	>40	>1300[A]

□ TSH not available. Previous TSH = 1.96 mU/L (182 days before); next TSH = 159.68 mU/L (583 days after, corresponding to time-point C, same day of thyroid dysfunction onset).

Hypothyroid patients

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time							Time-point C: Post thyroid dysfunction						
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
15	2.34	0.26[1]	0.72	-1.44	0.6	688[A]	NA	NA	NA	NA	NA	NA	NA	0	6.58	0.10[1]	1.10	15.79	NA	377[A]
37	2.26	0.09[4]	1.54	-31.97	NA	68[A]	337	NA	0.07[4]	1.63	-9.95	NA	NA	6	35.46	0.10[4]	0.63	-12.25	<1	>1300[A]
44	1.34	0.07[4]	1.09	7.16	<1	21[B]	NA	NA	NA	NA	NA	NA	NA	0	9.18	0.25[4]	0.86	-6.08	NA	377[B]
33	NA	NA	NA	NA	NA	NA	28	0.18	0.87[5]	2.33	66.27	NA	>1300[A]	159	5.39	0.68[5]	1.21	21.56	>40	>1300[A]
31	NA	NA	NA	NA	NA	NA	203	1.49	0.10[5]	1.26	-7.90	0.8	672[A]	672	*	0.08[5]	0.94	2.44	NA	>1000[B]
29	NA	NA	NA	NA	NA	NA	210	1.34	0.24[5]	1.17	-27.18	0.5	37[A]	224	5.26	0.90[5]	1.21	53.82	>40	496[A]
32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	80.42	0.78[5]	0.99	-11.59	20.7	>1300[A]

* TSH not available, but likely within the normal range, considering the evidence of stable euthyroidism under levothyroxine treatment for nearly 4 years post-ALTZ.

NO-TD patients

ID	Time-point A: Pre-ALTZ						Time-point B: Latest time post-ALTZ					
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
13	1.63	0.15[1]	1.13	13.88	0.85	<10[A]	1.84	0.05[1]	1.03	30.92	0.3	<2[B]
25	1.30	0.23[3]	0.82	8.99	<1	<2[B]	0.90	0.19[3]	1.15	26.62	<0.9	<2[B]
17	0.82	0.21[2]	1.04	0.58	<1	<2[B]	1.54	0.39[2]	1.08	-2.39	<0.9	<2[B]
19	1.22	0.25[5]	0.75	-2.13	NA	<2[B]	★	0.33[5]	0.88	-1.84	<0.9	<2[B]

★ TSH not available, but likely within the normal range, considering the evidence of stable euthyroidism for 5.9 years post-ALTZ.

Summary of autoantibodies to the thyrotropin receptor (TRAb), autoantibodies to thyroid peroxidase (TPOAb) and thyroid stimulating hormone (TSH) status among patients positive for in-house TRAb assays (ihTRAb+). Only patients resulted ihTRAb+ in at least one time-point are represented.

White cells = negative TRAb/TPOAb results. Colored squares represent positive results: azure = TRAb detected by flow cytometry (FC-TRAb); green = stimulating TRAb detected by luciferase bioassays (LB-TSAb); purple = blocking TRAb detected by luciferase bioassays (LB-TBAb); grey = TRAb detected by automated systems (AutTRAb); yellow = TPOAb (automated assay).

ALTZ = Alemtuzumab. GD = Graves' disease. ID = patient's identification number. NA = Not Applicable or Not Available.

TSH normal reference range varies between 0.30 – 4.4 mU/L and 0.35 – 5.5 mU/L, depending on the assay used and the date of test (see supplemental table 1).

$^{\alpha}$ = Time (days) before onset of thyroid dysfunction

$^{\beta}$ = Time (days) after onset of thyroid dysfunction

Reference Ranges and Positivity Cut-offs

AutTRAb (IU/L) reference ranges: negative <1, borderline 1–1.5, positive >1.5 (until January 2014); negative <0.9, borderline 0.9–1.6, positive (from February 2014 onwards)

^{A,B} = TPOAb (U/ml) reference ranges: ^{A} = negative <60, positive ≥60 (until May 2010); ^{B} = negative <6, positive ≥6 (from June 2010 onwards)

FC-TRAb positivity cut-offs. Samples have been tested in 5 different sets of experiments, each producing slightly different mean of greatest differences in fluorescence intensity between the two histograms (D) and relative standard deviation (SD) among pooled controls. Samples were considered positive if $D_{sample} > D_{controls} + 2$ SD.

^{1} = Set 1 $D_{controls} + 2$ SD = 0.26

^{2} = Set 2 $D_{controls} + 2$ SD = 0.30

^{3} = Set 3 $D_{controls} + 2$ SD = 0.42

^{4} = Set 4 $D_{controls} + 2$ SD = 0.21

^{5} = Set 5 $D_{controls} + 2$ SD = 0.32

LB-TSAb positive if stimulation index (SI) >1.5.

LB-TBAb (%) positive if inhibition index (InI) >20%.

SUPPLEMENTAL MATERIAL

Supplemental Table 1: Automated Laboratory Assays and Reference Ranges for TPOAb, FT4, FT3 and TSH

TIME PERIOD	ASSAY	REFERENCE RANGES			
		TPOAb (U/mL)	TSH (mU/L)	FT4 (pmol/L)	FT3 (pmol/L)
From 1/1/2006 To 31/5/2010	Siemens ADVIA Centaur	<60	0.35 – 5.5	10.0 – 25.0 (from 1/1/2006) 9.8 – 23.1 (from 21/5/2009)	3.5 – 6.5
From 01/06/2010 To current	Abbott Architect	<6	0.30 – 4.40 0.35 – 5.0 (from 29/6/10) 0.30 – 4.4 (from 31/1/2014)	9.0 – 19.1 9.2 – 21.0 (from 31/1/2014) 9.0 – 19.1 (from 5/11/2014)	2.6 – 5.7

TSH = thyroid stimulating hormone. FT4 = free-thyroxine. FT3 = free-triiodothyronine.

Supplemental Table 2: TRAb and TPOAb predictive values, sensitivity and specificity at time-point A

		Sensitivity	PPV	Specificity	NPV
TD versus NO-TD	ihTRAb	5/16 (31.2%)	5/5 (100%)	14/14 (100%)	14/25 (56.0%)
	TPOAb	5/16 (31.2%)	5/5 (100%)	14/14 (100%)	14/25 (56.0%)
	ihTRAb and/or TPOAb	7/16 (43.8%)	7/7 (100%)	14/14 (100%)	14/23 (60.9%)
HYPER versus NO-TD	ihTRAb	1/10 (10.0%)	1/1 (100%)	14/14 (100%)	14/23 (60.9%)
	TPOAb	2/10 (20.0%)	2/2 (100%)	14/14 (100%)	14/22 (63.6%)
	ihTRAb and/or TPOAb	2/10 (20.0%)	2/2 (100%)	14/14 (100%)	14/22 (63.6%)
HYPO versus NO-TD	ihTRAb	4/6 (66.7%)	4/4 (100%)	14/14 (100%)	14/16 (87.5%)
	TPOAb	2/10 (50.0%)	3/3 (100%)	14/14 (100%)	14/17 (82.3%)
	ihTRAb and/or TPOAb	5/6 (83.3%)	5/5 (100%)	14/14 (100%)	14/15 (93.3%)

TD = thyroid dysfunction; NO-TD = absence of thyroid dysfunction; HYPER = hyperthyroidism; HYPO = hypothyroidism; ihTRAb = TRAb measured with in-house assays; PPV = positive predictive value; NPV = negative predictive value.